IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of

BROWN et al — Atty. Ref.: 4598-2; Confirmation No. 2642

Appl. No. 10/723,420 — TC/A.U. 1614

Filed: November 26, 2003 — Examiner: Royds, L.

For: BIOLOGICALLY ACTIVE METHYLENE BLUE DERIVATIVES

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION

I, DAVID M. LEWIS, do hereby declare and state as follows:

1. I have a PhD degree in Colour Chemistry and I have held the position of Professor and Head of the Department from 1987 to 2004, and Emeritus Professor from 2004 to the present time, in the Department of Colour Chemistry at the University of Leeds. In my 40 years experience in industry and academia as a dye chemist, I have gained an in-depth knowledge of the chemistry of dyes and their applications. My research experience includes studies of the relationships between dye structure and dye properties, the mechanistic and technological aspects of dye uptake by natural and synthetic polymers, dye photochemistry, modified polymers and bactericidal agents. A copy of my curriculum vitae is attached.

2. I have reviewed the contents of U.S. Application No. 10/723,420 and the claims presently on file, specifically, claims 77-79, 84, 89-91, and 98 - 100. I have also reviewed the

Office Action dated 25th June 2007, and understand that the Examiner has rejected the claims on the basis that they would have been obvious over various prior art references [Note 1 (b) - (h)]. The Examiner suggests that the prior art references which are directed to the use of Methylene Blue which is of identical core structure to the presently claimed compounds would had rendered the claims obvious because the skilled person would have reasonably predicted that the present compounds would share similar pharmacologic properties with those of Methylene Blue due to their structural homology. Further, the Examiner suggests that that the skilled person would have expected the present compounds to have exhibited bactericidal activity against the claimed bacteria the same or substantially similar to that exhibited by Methylene Blue.

3. The cationic dye Methylene Blue has been known since 1876, and since the end of the 19th century has been investigated as an antimicrobial agent for several therapeutic applications [see for example **Ref. 1**, p.99]. It is notable that the photoantimicrobial activity of Methylene Blue is reported as early as 1930 [**Ref. 2** p.17), and yet despite the obvious rewards of producing improved analogues. I am not aware of any report of any higher alkyl homologues (ethyl, propyl, butyl etc.), or homologues with other hydrophobic side chains, ever having been investigated as antimicrobial agents prior to the filing of U.S. Application No. 10/723,420. This suggests that there were good scientific reasons why researchers were not led to investigate these higher hydrophobic homologues prior to the present invention. M. Wainwright in his review of photodynamic therapy (**Ref 1**, p. 100) states: *"Phenothiazinium dyes, usually based on the Methylene Blue structure, are eminently suited to use as biomedical photosensitisers. ... Unfortunately the optimal nature of the lead compound* (i.e. Methylene Blue) *has often discouraged the search for improved analogues."*

It is my opinion that conventional scientific thinking on the mechanism of cationic dye staining of polymers (i.e. microorganisms), which is one of attraction between oppositely charged ions [see for example **Ref. 3**, pp 2-3], has in the past led the skilled person away from using more hydrophobic higher homologues of Methylene Blue as photo-antibacterial agents of equivalent activity to Methylene Blue. In my opinion, existing knowledge would have made it unobvious that such Methylene Blue homologues would have had any advantages over Methylene Blue itself, and indeed the literature would have suggested that there would be a greater likelihood of such homologues having therapeutic properties inferior to those of Methylene Blue. My reasons for this opinion are as follows:

It was generally accepted that photo-antimicrobial cationic dyes such as Methylene Blue worked by first staining biological components of the cell, and then, after exposure to light, generating singlet oxygen which attacked cell constituents and caused rapid cell death. [See for example M. Wainwright, **Ref. 4**, p. 351 " *If a known synthetic photosensitizer such as toluidine blue could be seen to stain a pathogenic organism sufficiently, then it should be possible to destroy that organism via subsequent irradiation.*"; M.Wainwright, **Ref. 2,** p 14. "*It is this work which underlies the principle of photodynamic antimicrobial action—if a live microbe could be demonstrated selectively with a vital stain such as methylene blue, it should be possible to destroy the stained microbe on illumination*"]. Thus, efficient adsorption of the dye onto the cell components in contact with the dye solution, i.e. cell staining, was seen to be one of the most important characteristics of a therapeutically effective photo-antimicrobial agent.

This selective ***cell staining*** by cationic dyes (such as Methylene Blue) has been used for many years [see for example **Ref. 4,** page 355] in the histological study of microorganisms. ***Cationic dyeing of textiles*** operates by an identical process to cell staining, and similarly involves selective uptake of a cationic dye from solution by an anionic polymer, such as protein fibers (e.g. wool, silk) and polyacrylic fibres modified with anionic groups. Thus, the effectiveness of a cationic dye as a photo-antimicrobial agent was understood to be related to its effectiveness both as a biological stain and as a dye for anionic fibers.

It was also understood [see for example **Ref. 5**] that cell staining with cationic dyes was most successfully achieved using an aqueous solution of the dye. Almost all microbiological stain solutions based on ionic dyes use water as the preferred solvent. Ethanol is sometimes used, but gives poorer uptake **[Ref. 5]**. Water was viewed as the ideal solvent for delivery of a cationic photo-antimicrobial dye to microorganisms in a therapeutic situation for the following reasons:

(a) Uptake of a cationic dye at the negatively charged cell surface involves ion exchange, in which cations on the biopolymer (H^+ Na^+ etc.) are replaced by the dye cation. The dye cation then becomes paired with an anionic group on the surface. **[Ref. 3,** pages 2-3**].** Water is a unique solvent, and its exceptionally high dielectric constant and solvating power ensures dissociation of ion pairs. The ion exchange process thus proceeds with much greater facility in water than in organic solvents.

(b) Alternative organic solvents (usually ethanol) are often unsuitable for staining with cationic dyes, and give poorer uptake when the staining mechanism is ionic in nature **[Ref. 5]**.

(c) An additional important factor which made water the solvent of choice for photo-antimicrobial dyes is that it is the only solvent that could be used safely in undiluted form for therapeutic application to living tissue. Organic solvents (such as ethanol) could only be used if diluted with water to low concentrations because of their inherent adverse effects on the host tissue.

Thus the skilled person developing cationic dye photosensitizers for therapy would have been led to water as the ideal vehicle for delivering these dyes to the target microorganism.

In staining or dyeing, if a cationic dye is to be adsorbed from an aqueous solution onto a cell or textile fiber surface, then good water solubility had long been regarded as an important prerequisite, as the rate of adsorption was expected to be dependent on the concentration of the dye in the aqueous phase. Thus, it had long been accepted that cationic dyes for textile dyeing needed to have good water solubility so that solution concentrations conducive to rapid dye uptake could be used. Dyes that had low water solubility as a consequence of hydrophobic side chains were expected to produce only weak aqueous solutions when fully saturated and such weak solutions were expected to result in unacceptably slow uptake rates. This perceived good water solubility requirement is clearly indicated by an examination of the **Colour Index [Ref. 6]** which lists all known dyes that have ever been commercialized. Consideration of the 3rd Edition (1971) shows that more than 150 synthetic cationic dyes of known structure had been commercialized up to that time, and all of these had the common characteristic of good water solubility. Representative examples from different chemical classes are: the oxazine Basic Blue 3 (solubility 3g/100ml), the rhodamine Basic Red 1 (solubility 2 g/100ml), the thiazole Basic

Yellow 1 (solubility 2g/100ml; the triphenylmethane Basic Violet 1 (solubility 5g/100ml), and Methylene Blue itself (Basic Blue 9) (solubility 5 g /100ml). [Solubility data taken from **Ref. 7**]. All N-alkylated cationic dyes listed by the **Colour Index** had alkyl groups no longer than two carbon atoms and most had methyl groups, clearly suggesting the need for hydrophobic side chains to be kept to a level where water solubility was not compromised. The only exception is the azine dye CI 11090 which has a dihydroxypropyl side chain, and in this case the two adjacent hydrophilic hydroxy groups are present to counteract the insolubilising effect of the longer propyl chain. Focusing attention on the phenothiazinium class, the **Colour Index** shows that just nine Methylene Blue type structures have been commercialized since 1876 to the present day. All these compounds are anticipated to be very water soluble, as none have alkyl chains longer than ethyl. Water solubility data could be found for six of these **[Ref. 7]**, and as expected, values are high and fall in the range 1-5 g/100 ml.

By analogy, cationic dyes for microbial staining would have been expected to have good water solubility, and this is also confirmed by the commercial record. The Sigma Aldrich catalogue **[Ref.8]** lists the widest range of microbiological stains in the world, many certified by the Biological Stains Commission, and includes six cationic phenothiazinium dyes among the 300 or so stains listed. These six (Methylene Blue, Azure A, Azure B, Azure C, Toluidine Blue and New Methylene Blue) are also listed in the **Colour Index**, and as noted had water solubilities in the range 1-5 g/100ml.

The marked absence of longer chain hydrophobic analogues of Methylene Blue from the historical commercial textile dye record and from microbiological stain listings could not have been due to any lack of availability of such compounds, since synthetic routes to such homologues had been readily available for more than 130 years (see for examples references 9

and 10). The absence of these compounds would have been due to their actual or perceived inferior dyeing and staining properties compared to Methylene Blue itself, such inferiority having been expected to stem from their lower water solubility.

The pronounced effect of increasing hydrophobicity on water solubility in the Methylene Blue series is exemplified by the following data for alkyl derivatives:

Solubilities in water at ca. 25 °C:

Methylene Blue	*~ 5 g / 100ml*
Ethylene Blue	*~ 3.9 g / 100ml*
Propylene Blue	*~ 0.01 g/ 100ml*
Butylene Blue	*~ 0.015 g /100ml*
Pentylene Blue	*~ 0.0001 g/100ml*

[Data for Methylene Blue from Ref. 7. Data for other dyes measured by Photopharmica]

Thus, the propyl and butyl derivatives show an approximately 500 fold decrease in solubility compared to Methylene Blue, and the pentyl derivative a four orders of magnitude reduction. Such low water solubilities would have been expected to preclude these compounds from having any technical value as textile dyes, and would have suggested that they would had little value as aqueous biological stains. It was therefore most surprising that these compounds had such dramatically greater photo-antimicrobial activities than Methylene Blue and Ethylene Blue. Thus, the relative cell kill efficiencies against *E. coli* are:

Methylene blue	**3.5**
Ethylene Blue	**1.7**
Propylene Blue	**8.1**
Butylene Blue	**52,000**
Pentylene Blue	**195,000**

This major enhancement in photo-antibacterial properties is even more surprising when one takes into account that there is a small decrease in activity when the homologous series is ascended from Methylene Blue to Ethylene Blue.

In conclusion, it is my opinion that long-established conventional thinking on the relationship between the water solubility of a cationic dye and its effectiveness both as a textile dye and as a biological stain would had led the skilled person away from considering more hydrophobic higher homologues of Methylene Blue, such as those claimed in US Appln. No.10/723420 as useful photo-antimicrobial dyes. Convention would have taught that such an increase in molecular size, where the increase in hydrophobicity resulted in a significant decrease in water solubility of the dye, would be detrimental to dye uptake and thus detrimental to the observed photo-antimicrobial activity. On this basis the skilled person would not had been motivated to consider higher homologues. Additionally, even if an artisan were to have considered higher homologues he/she would have expected poorer results rather than the significantly improved results obtained in the present invention. This is particularly the case if one considered the activities of Methylene Blue and Ethylene Blue. A researcher systematically investigating the effect of increased alkyl chain length and increasing hydrophobicity on activity would have found that extending the chain length from methyl to ethyl resulted in a decrease in activity, and so would have been dissuaded from examining longer chains.

There has been a long felt medical need for powerful antimicrobial drugs which operate by a mechanism different from that of antibiotics: drugs that could be used topically on

infected wounds and ulcers, had broad spectrum activity even against "superbugs", and are unlikely to lead to drug-resistant strains of microorganism. Despite the fact that photo-antibacterial therapy has long been considered a way of achieving these objectives, none of the extensive research that has taken place prior to U.S. Application No. 10/723,420 succeeded in producing new photosensitizers with the high photo-antimicrobial activities of the present compounds.

* * * * *

I hereby declare that all statements made herein of my own knowledge are true and that statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Further, declarant sayeth not.

Signed this 14th day of August, 2007.

David M Lewis

David M. Lewis

NOTE 1:

Prior art cited in Office Action dated 25th June 2007:

(a) Y. Mazur et al. *"Phenothiazinium salts and their use for disinfecting aqueous effluents".* U.S. Patent No. 5,220,009; (1993).

(b) S J Wagner *"Decontamination of whole blood and cellular components by phenothiazin-5-ium dyes plus light". WO*/91/16911 (1991)

(c) M Wainwright et al *"Photobactericidal activity of phenothiazinium dyes against methicillin-resistant strains of Staphylococcus aureus".* FEMS Microbiology Letters, vol.160, No. 2 (1998) pp 171-181.

(d) E. Shanbrom *"Microbicide treated polymeric materials."* U.S. Patent No.6,183,764;(2001).

(e) M. Wilson and W. Harvey, *"Laser treatment".* U.S. Patent No. 5,611,793 (1997)

(f) M A Biel . *"Photodynamic cellular and acellular organism eradication utilizing a photosensitizing material and surfactant".* WO 01/62289 (2001).

(g) R K Chowdhary and D Dolphin. *"Supports for photosensitizer formulations"* U.S.Patent Application Publication 2002/0061330 (2002).

(h) ***The Merck Index*, Monograph 5979 (1989).**

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4. Mark Wainwright "Non-porphyrin Photosensitizers in Biomedicine" ***Chemical Society Reviews***, 1996, 351-359 .

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CURRICULUM VITAE

Professor David M. Lewis, BSc, PhD, FRCS

Business Address: Department of Colour Chemistry
University of Leeds
Leeds, LS2 9JT
UK

Telephone: (+44) (0)113 343 2931
Fax: (+44) (0)113 343 2947

Research experience: 40 years experience in industry and academia, in the general areas of dye chemistry and dye application technology. 215 refereed publications in the fields of dye chemistry, dyeing technology, dye photochemistry, polymer chemistry, analytical chemistry and ink technology. 38 patents in related fields.

Current positions:

- Emeritus Professor, Department of Colour Chemistry, The University of Leeds, Leeds LS25 9JT, UK.
- Research Director, Inovink Ltd.
- Chief Scientific Officer, Perachem Ltd.

Previous employment history:

1966 – 1978: Development Officer - Principal Development Officer, International Wool Secretariat (IWS) Ilkley. Development of new dyeing and chemical finishing processes for wool and the part supervision of chemical aspects of the Wool Foundation Northern Hemisphere sponsored research scheme.

Jan 1978 – April 1979: Senior Research Scientist, CSIRO, Division of Textile Industry, Geelong, Australia. Development of metallisable, sublimable disperse dyes for the transfer printing of wool and wool-blend fabrics.

April 1979 – April 1987: Principal Development Scientist, IWS. Coloration and chemical finishing developments..

April 1987-August 2003. Professor and Head of Department of Colour Chemistry, University of Leeds.

August 1998 – August 2003: Head of Resource Centre, School of Physical Sciences, University of Leeds.

Honours and Achievements:

Silver medal of the Society of Dyers and Colourists for Technological Achievement, March 1985.

Chairman of the West Riding Region, Society of Dyers and Colourists, March 1986 – March 1988.

Fellowship of the Royal Society of Chemistry, awarded in 1984.

Visiting Professorships in China at the North West Textile Institute 9Xian), the University of Heilongjiang (Harbin) and the Technical University of Wuhan.

Chairman, International Association of Textile Chemists and Colourists Studentship Committee from June 1989.

Gold Research Medal, Worshipful Company of Dyers: for papers published on Reactive Dyes (January 1992).

President, Society of Dyers and Colourists, April 1993 – April 1994.

Gold Medal of the Worshipful Company of Dyers, April 2005.

Millson Award for Invention, by the American Association of Textile Chemists and Colourists ,2005.

Accreditation/Validation Experience:

BSc (Hons) Course Validator at Scottish College of Textiles, April 1987.

External Examiner, BSc (Hons) Applied Chemistry, Scottish College of Textiles, April 1987 – September 1990.

BSc (Hons) Course Validator at Hong Kong Polytechnic, April 1991.

External Examiner, BSc (Hons) Textile Chemistry, Hong Kong Polytechnic (1991/92 – 1994/95).
Science assessor for CSIRO review group, Geelong, Australia – March 2007.

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